PROTEOLYTIC ENZYMES IN *CHAOS CHAOS*

by

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CONTENTS

1. INTRODUCTION

In 1936, HOLTER and KOPAC (17) studied the distribution of dipeptidase in centrifuged specimens of *Chaos diffluens*. In confirmation of previous results with other materials (12), it was found that peptidase activity was independent of all cytoplasmic inclusion bodies that could be stratified by the centrifugal forces employed (3.800 × g). There is abundant evidence in the literature that peptidase is a very ubiquitous enzyme, occurring in large amounts in all kinds of cells and tissues. As far as its cytological localisation has been studied it has always been found to be very diffusely distributed, in the

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hyaloplasm or in very small granules, and possibly in both. The physiological function of this enzyme remains still largely unknown and although it has been often assumed in a vague way that it must be somehow connected with protein synthesis, no definite evidence for this has been obtainable.

In our investigations on the cytochemistry of amoebae we have for a long time been anxious to compare the distribution of peptidases with that of the proteinases which attack high-molecular proteins, since we thought that such a study might throw some light upon the physiological function of both groups of enzymes. There were, however, technical difficulties. The determination of peptidase at a cytochemical level by means of the microtitration technique of LINDERSTRØM-LANG and HOLTER (20) is comparatively easy, but it soon turned out that the proteinase activity of amoeba cytoplasm was so low that it could not very well be determined by means of titration methods.

This difficulty was overcome by the colorimetric micromethod described by DUSPIVA (9), which could easily be adapted to the dimensions necessary for the study of amoebae.

Another difficulty which has forced us to postpone our investigation for some years was that of correlating enzyme determinations with the size of the cell fragments used. Recently, however, two methods have been developed which can be used to measure the size of cells of this magnitude. One is the determination of cell volume (HOLTER (14)), the other is the determination of the reduced weight in the Cartesian diver balance by ZEUTHEN (25). By means of these methods we are now able to measure "specific" enzyme activities (per unit of reduced weight or volume) of centrifuged fragments of amoebae and thus to study the enzyme distribution in a more quantitative way than previously. In the present study only the reduced weight method has been used in measuring the size of cells, the reason being that the amoebae are less damaged by the diver balance weighings than by the volume determinations.

The following is an account of our results with dipeptidase and catheptic proteinase. Since these two enzymes have not previously been studied in *Chaos chaos* we had also to investigate some of their general properties (PH-optimum etc.) as far as this was necessary for the quantitative determination of activity. The results of these preliminary experiments will be included in the following report.

2. MATERIAL AND METHODS

2.1. Material.

The strain of *Chaos chaos* was the same as the one employed in previous investigations (1, 2). The organism had been maintained in culture in this laboratory since 1938, without any possibility of renewal owing to the war, and a new shipment from U.S.A. was therefore obtained in 1946 and the fresh specimens were compared with the old stock. No demonstrable difference in morphology or viability could be detected.

The cultures were grown in PRINGSHEIM solution as previously (1, 2) described. If nothing else is stated, PRINGSHEIM solution was also the medium in which all experiments described in this paper were performed. Before weighing, centrifuging etc. the amoebae had usually been starved for 24 hours to make their contents more homogeneous.

Recently there was a discussion about the proper zoological name for the organism used in this investigation (19, 21, 24). The names proposed are *Chaos chaos, Chaos carolinensis* and *Pelomyxa carolinensis*. Not being taxonomists, we do not want to participate in this discussion but we should like to draw attention to one point which so far has not been mentioned. By the criteria of cytology and cell physiology, the similarity between *Chaos chaos* and *Chaos diffluens* (*Amoeba proteus*) is so great that they ought to be included in the same genus. Therefore, while we do not want to interfere with the deliberations of the taxonomists regarding the relative merits of the generic names proposed, we should consider as unsatisfactory any decision which does not classify the two organisms within the same genus.

2.2. Homogenates of amoebae.

Since the preliminary experiments on the general properties of the enzymes investigated had to be carried out with homogeneous solutions, some means of preparing such solutions had to be found. The tissue homogenizer of POTTER and ELVEHJEM (22) was therefore reduced to a suitable size, and was found to work quite satisfactorily.

The amoebae homogenizer (fig. 1) consists of a lucite tube of the dimensions given in the figure. The lucite pestle is turned on a lathe and ground to fit the lower, narrower, part of the tube with a play of about 20 μ.

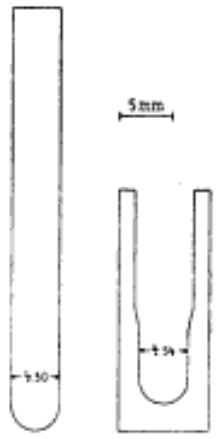

Fig. 1. Amoeba homogenizer.

In the first specimens the pestle had spiral grooves on its lower end, but since the grooves did not seem to improve the efficiency and certainly made the cleaning much more difficult, they were later omitted.

The homogenizer is worked in the following way: About 3 µl of culture solution containing 10–30 amoebae is placed in the bottom of the lucite tube by means of a braking pipette (13). To this is added 3 µl of phosphate-glycerol (40 % glycerol, 0.125 M with regard to phosphate buffer, PH 7.4). The pestle is introduced, avoiding air bubbles, and the amoebae are ground by rotating the pestle by hand and at the same time drawing it about 1 mm up and down the tube. After about 1 minute's grinding the pestle is lifted into the wider part of the tube and a suitable volume (generally about 100 µl) of weaker phosphate-glycerol (20% glycerol, 0.005 M phosphate) is added, at the same time rinsing the pestle. When samples from this homogenate are taken by means of a micropipette, it is advisable to stir the suspension during the pipetting by means of one of the magnetic stirrers used in microtitration.

In most of the subsequent experiments the number of amoebae and the dilutions were so adjusted that a 7 µl sample of the homogenate corresponded to 0.5–1 amoeba.

2.3. Centrifugation.

The centrifuge used for the stratification of our amoebae was the microscope centrifuge described by E. N. HARVEY (10) in which the object can be observed during centrifugation. HARVEY'S original model, however, is intended for suspensions of a great number of cells so that in his case a stationary field of vision was sufficient. Since we had to keep track of one single cell during its centrifugation, the centrifuge had to be modified so as to permit focussing and a certain lateral movement of the field of vision while the centrifuge was running. This was accomplished by providing for a slight inclination of the microscope tube against the optical axis of the instrument and by a micrometer screw which changes the distance between the lenses of the eye-piece. The total area that can be controlled by this device has a diameter of 2.5 mm, the range of focus is 0.5 mm.

A detailed description of the apparatus cannot be given here. It was built by the firm H. STRUERS Chemiske Laboratorium, Copenhagen. The maximum speed is 14.500 r. p. m., corresponding to a centrifugal force of 21.000 g. Two objectives in the rotor can be used alternatively by turning a prism in the centre of the rotor. Their magnification is 10X and 30X, corresponding to total linear magnifications of 100X and 300X. The samples are placed in cuvettes of the type shown in fig. 2, consisting of a glass plate with a longitudinal

groove, 2 mm wide and 0.5 mm deep, covered by a coverslip which is cemented on by Canada balsam.

For centrifugation the amoeba is placed in the cuvette on a cushion of gum arabic solution. For this purpose 2–3 µl of a 20% solution of dialyzed gum arabic is placed in the bottom of the cuvette and on top of this the amoeba is introduced together with 4–6 µl of culture solution. The slight mixing involved in this operation is usually sufficient to establish a suitable gradient of specific gravity, on which the amoeba rests during centrifugation. The relative amounts of gum arabic and culture solution must of course be so adjusted that the amoeba comes within the field of vision of the microscope. To remove the centrifuged amoeba, the cuvette is filled up with culture solution, inverted and placed in a suitable vessel filled with the same solution. The amoeba slides out by its own weight.

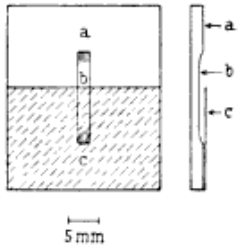

Fig. 2. Centrifuge chamber seen from front and from side. a: glass plate. b: ground depression. c: coverslip.

Since the amoebae had to stand a certain amount of handling after centrifugation they were stratified only to a degree where recovery was still possible. The time and speed of centrifugation was varied according to the appearance of the individuals in the microscope centrifuge. Regarding the general appearance of centrifuged *Chaos chaos* and the arrangement of the strata the paper by ANDRESEN (1) should be consulted. Generally a centrifugation of 30-40 minutes at 1.000 × g was sufficient. Under these conditions, and at room temperature, remixing of the cytoplasm begins immediately when the centrifugal force ceases to act, and to prevent this the centrifuging and the subsequent operation were done in a cold room at 2°. To obtain a clear-cut stratification the amoebae must at the beginning of centrifugation have an approximately spherical shape. They usually round up by themselves 15-30 minutes after transfer to the cold room. If the cooling alone is not sufficient, rounding up can be aided by manipulating with a blunt glass needle.

2.4. Operation.

After centrifugation the amoeba is removed from the cuvette and transferred to a drop of ice-cold culture solution on a microscope slide. Then it is cut at the desired level by means of a glass needle under a dissecting microscope. Since the shape of the centrifuged amoeba usually is not ellipsoid but rather like a pear with its thick end at the heavy pole, the cutting

often results in somewhat small light and bigger heavy halves[1)], even if the operation was intended to yield approximately equal portions. But as the halves are weighed and analyzed individually, this does not matter.

2.5. Weighing.

The amoebae were weighed on Cartesian diver microbalances in the manner described by ZEUTHEN (25). The sentivity of the balances used was in the neighbourhood of $\Delta p = 5–15$ cm BRODIE solution per μg reduced weight (in most experiments 10 cm). In all subsequent experiments the mass of the amoebae will be expressed in μg reduced weight.

2.6. Cytolysis.

Weighed amoebae or portions of amoebae were used for the enzyme determinations. The purpose of these determinations was to find the total amount of enzyme acting on a certain substrate under reproducible and comparable conditions, and it was therefore necessary to make sure that all the enzyme present was actually in contact with the substrate added. For this reason we ran a series of experiments in which the influence of cytolysis on the enzymatic activity was studied.

In one series cytolysis was brought about by mechanical means in the manner described by DOYLE (8). This method consists of transferring the object (in DOYLE's case sea urchin eggs) with a very small amount of water to a 2 × 2 mm square of coverslip glass, teasing it to pieces by means of glass needles under the microscope, and introducing it together with the coverslip into the enzyme digestion tube.

The other way of causing cytolysis was by means of a deter- gent , which is used for erythrocyte hemolysis and is sold under the name "657 L" by the "Société Belge de l'azote et des produits chimiques du Marly". This product "657 L", which is short for "657 LABAZ" is the decaglycollic ether of p-(2-trimethyl ethyl) phenol. It has been synthesized in the pharmaceutical research laboratories of the "Société Belge de l'Azote". It is obtained in a 5 % aqueous solution. When amoebae were introduced into various mixtures of this stock solution with PRINGSHEIM solution, they

[1] This inequality becomes much greater if we compare not the volumes but the reduced weights, owing to the difference in specific gravity of the fragments.

disintegrated within times roughly proportional to the degree of dilution. The disintegration consisted mainly of a dissolution of the ectoplasmic layer, leaving the amoeba as a shapeless heap of cytoplasmic debris at the bottom of the vessel, easily dispersed by stirring. For the enzymatic experiments a dilution of 1:20 was used, causing cytolysis within 20-30 minutes.

2.7. Peptidase determination.

The peptidase determinations were carried out as previously (16) described. The substrate was d, l-alanyl-glycine in 0.2 M solution without addition of activator. pH 7.4, incubation temperature 40°, incubation time usually 3 hours. 7 µl of amoeba homogenate or the amoeba itself in 7 µl of phosphate-glycerol (20% glycerol, 0.005 M phosphate pH 7.4) were in a microtube mixed with 7 µl of substrate and incubated; the reaction was terminated by the addition of 30 µl of 0.06 N alcoholic HCl and the enzymatic cleavage determined by titration with 0.06 N alcoholic HCl. Blanks consisted of microtubes containing all reagents except the amoebae. Preliminary experiments had shown that HCl-consumption of the amoeba-cytoplasm itself was at an average 0.07 µl 0.06 N HCl per amoeba. This value was so low compared with the cleavage of substrate that it could be neglected.

Enzymatic cleavages are expressed in µl 0.06 N acid. Regarding the validity of this unit, compare section 3.13. Total splitting of the l-component of substrate corresponds to a titration value of ~ 12 µl.

2.8. Proteinase determination.

The method described by DUSPIVA was used (9) with a few minor modifications destined to adapt the method to the dimensions of the available photometer.

2.81. Chemical procedure.

Substrate: 6 µl of a 1:1 mixture of Casein-urea substrate and pH 3.75 buffer (phosphate-citrate-ammonia) prepared according to DUSPIVA. The enzyme sample consisted of 3 µl of amoeba homogenate or an amoeba sample plus 3 µl glycerolphosphate as described for peptidase.

After 2-4 hours incubation af 40° the reaction was stopped by addition of 30 µl water and 60 µl trichloracetic acid (0.3 M), and the mixture stored in the ice-box. Next day the mixture was centrifuged (5 min, at 1000 × g), 30 µl of the supernatant were removed, mixed with 30 µl 1 N NaOH and 18 µl Folin-Ciocalteu reagent and

the extinction read after 1-5 minutes. Since only 15 µl of liquid are used to fill the photometer cuvette it is obvious that the amounts and volumes given here could be still considerably reduced without any change in the technique.

2.82. Photometric procedure.

The instrument used was the Electronic photometer model 512 of the Photovolt Corporation, New York, in combination with a microscope. The light source was a Stufenphotometer lamp fed by a battery, and its light was by a suitable arrangement of diaphragms and lenses so adjusted that it formed a practically parallel beam with its focus about 3 mm above the level of the mechanical stage of the microscope. The diameter of the beam at its focus was appr. 0.3 mm.

The cuvette consisted of a piece of thick-walled pyrex capillary tubing of 1.2 mm bore, 5 mm high and ground parallel on both ends, at right angles to the bore. It was cemented into a microscope slide by means of picein and, when filled, closed on its upper end by means of a small square of coverslip glass. When a reading was to be taken, the cuvette was placed on the mechanical stage at such coordinates that the light beam occupied the centre of the capillary bore. In the measuring position the focus is halfway up the cuvette.

Having penetrated the cuvette the light is taken up by the low power objective of the microscope, forming an image at the level of a suitable arranged ground glass plate. The light intensity of this image is measured by the photometer, the ground glass plate being covered by the window of the photocell. Since we had a Leica microphotographic adapter at our disposal we mounted it between the microscope and the photocell. The use of its shutter for the illumination of the photocell and its lateral eyepiece for controlling the image of the cuvette contents is very convenient in practice, but it is, of course, not essential.

The arrangement described here gives plenty of light, so that there was no need to use the highest sensitivity afforded by the photometer. By increasing the sensitivity and cutting down the diameter of the light beam very much smaller cuvettes can be used if necessary, so that the scale of the whole method could easily be reduced by at least a power of ten, without any essential change.

The colour filter used for the measurements here described was the Stufenphotometer filter S. 72.

2.9. Terminology.

In the presentation of the experimental results, the following abbreviations and symbols will be used:

r. w. = reduced weight in μg.

A_{pe} = peptidase activity in μl 0.06 N HCl per hour.

A_{pr} = proteinase activity in "amoeba units". 1 amoeba unit corresponds roughly to the activity shown by 1 medium sized amoeba in 2 hours, and is empirically defined as the as the extinction caused by 2.9 µg tyrosine (= 3.15 tyrosine units) per 3.2 µl sample.

Pe = $\frac{A_{pe}}{r.w.}$ = specific peptidase activity (µl HCL per µg r.w. per hour)

Pr = $\frac{A_{pr}}{r.w.}$ = specific proteinase activity (am. un. per µg r.w.)

Q_{pe} = ratio of Pe values for two halves of one amoeba (comparison quotient). In the case of uncentrifuged amoebae the two halves a and b were so arranged that the largest, a, formed the numerator of the ratio Q_{pe} Pe_a/Pe_b. In centrifuged amoebae, the light (l) and heavy (h) halves were arranged so as to form the ratio $Q_{pe} = Pe_l/Pe_h$. Q_{pr} is the corresponding comparison quotient for proteinase activities. For centrifuged amoebae Q_{pr} Pr_l/Pr_h, for uncentrifuged $Q_{pr} = Pr_a/Pr_b$.

$\bar{x}$ = mean value of a quantity x.

S. D. (x) = standard deviation of x.

S. D.' (x) = $\frac{S.D.(x)}{\bar{x}}$ = relative S. D. of x (the use of this concept is explained in section 3.33).

S.E. (X) = standard error of x.

"strong" phosphate-glycerol = 40 % glycerol, 0.125 M phosphate buffer.

"weak" phosphate-glycerol = 20 % glycerol, 0.005 M phosphate buffer.

3. EXPERIMENTS

3.1. Peptidase.

3.11. pH optimum

Homogenate: 25 amoebae homogenized in 3µl "strong" phosphate-glycerol, added 30 µl 80 % glycerol. To 7 µl of this stock solution was added 57 µl of 0.05 M phosphate-glycerol of pH 6.0, 7.0, 7.5 and 8.0 respectively. To 7µl of these mixtures (corresponding

roughly to 1/2 amoeba per sample) were added 7 µl substrate, bringing the pH values of the final incubation mixtures to pH 6.3, 7.0, 7.5 and 7.8. Incubation time: 3 hours at 40°.

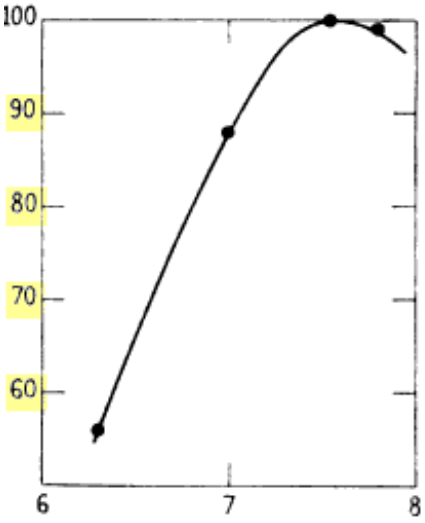

Fig. 3. p_H-optimum curve of peptidase activity. Abcissa: p_H. Ordinate: activity in per cent of maximum. Each point is the mean of 3 determinations.

Fig. 3 shows the result of two experiments. The curve gives the enzymatic splitting in per cent of the maximum value. The pH optimum of ~7.4 is the same as that previously found for a number of other objects. Accordingly pH 7.4 was maintained in all subsequent peptidase determinations.

3.12. Linearity of splitting with time.

Homogenate: 40 amoebae in 3µl (strong) +153 µl (weak) glycerolphosphate. 7 µl samples of this homogenate were mixed with 7 µl of substrate and incubated for various periods at 40°.

As shown by fig. 4, cleavage continues linearly until the proximity of total splitting. This agrees with previous findings for similar objects. Small deviations of incubation time from the planned value may therefore be corrected assuming linear progress of cleavage with time.

3.13. Cleavage as function of enzyme concentration.

Homogenate: 40 amoebae in 3 µl (strong) + 75 µl (weak) glycerol-phosphate. This stock solution was diluted in various proportions with glycerol-phosphate to give the relative en- zyme concentrations shown on fig. 5. Concentration 1.00 corresponds to 3.5 amoebae per sample of 7µl. Incubation in the usual proportions, incubation time: 3 hours.

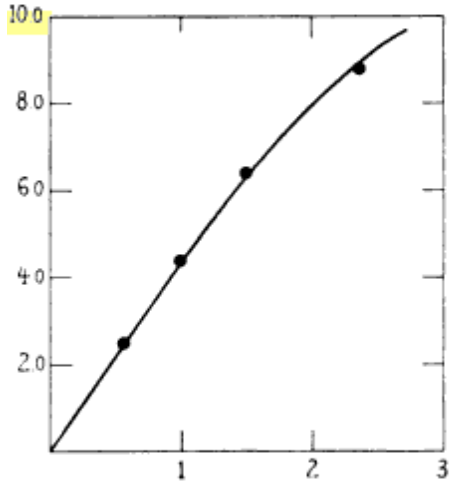

Fig. 4. Correlation between peptidase activity and time. Abcissa: hours. Ordinate: µl 0.06 N HCl. Each point is the mean of 3 determinations.

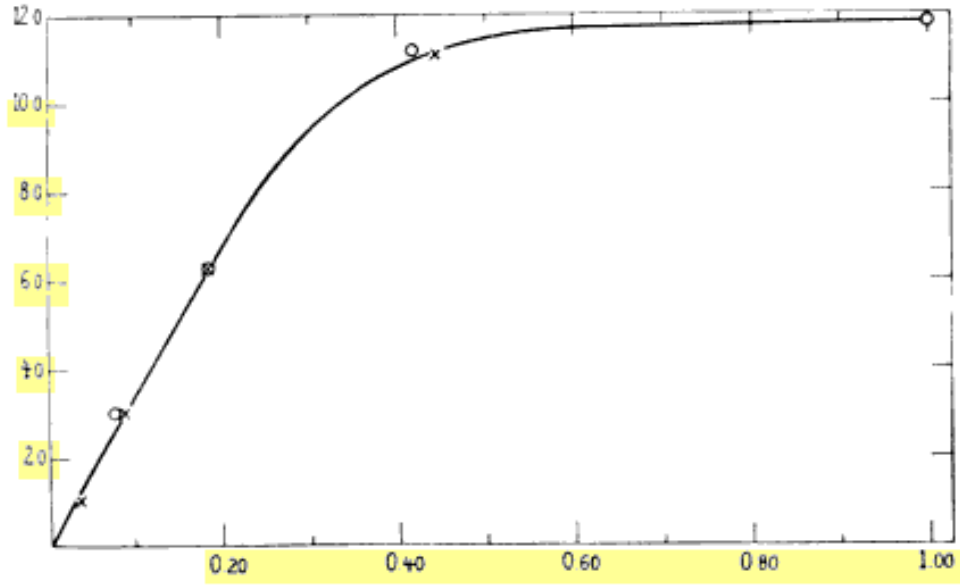

Fig. 5. Correlation between peptidase activity and enzyme concentration. Abcissa: relative enzyme concentration. Ordinate: µl 0.06 N HCl. ○ and × represent 2 different experiments. Each point is the mean of 3 determinations.

Fig. 5 shows the result of two experiments, graphically aligned. It is seen that the cleavage is a practically linear function of enzyme concentration until it approaches the total cleavage value of 12.0. It is therefore permissible to express the amount of enzyme present in a sample as µl HCl provided the determination is so conducted that the actual splitting observed remains between 0 and 8 µl.

3.14. Effect of grinding and cytolysis on peptidase activity.

These experiments involved essentially the comparison of different individual amoebae, treated and untreated. Such experiments meet the difficulty that the amoebae show very great individual variations in size and enzyme content, even if they come from the same culture. All animals had therefore to be weighed before comparison and the results had to be expressed per unit of reduced weight (= "specific activity").

In order to study the effect of homogenization the following experiment was performed: 15 amoebae were weighed (for practical reasons in two lots of 7 and 8 respectively) and were then transferred to 15 microtubes containing 7 µl glycerol-phosphate; substrate was added and peptidase was determinded in the usual

way. This gave us the r. w. and the peptidase activities of the animals comprised in the lot. ––15 amoebae of the same culture were weighed, combined, homogenized and the homogenate so diluted that every sample of 7 μl contained the homogenate of 1 (actually 1.04) amoeba. The peptidase activity of these samples was then determined . In- cubation was in both series 2 hours at 40°, pH 7.4.

Table 1.

Effect of homogenization on peptidase activity.

Lots 1 and 2 are given separately to show the deviation.

Untreated amoebae lot 1	8	1	5.66	0.71	2.72	3.84
Untreated amoebae lot 2	7	1	4.73	0.68	2.93	4.31
Untreated amoebae 1 + 2	15	1	10.39	0.69	2.81	4.06
Homogenate	15	1.04	11.13	0.77	3.23	4.19

As table 1 shows, the specific peptidase activity was practically the same in both lots. Therefore, if the homogenization is performed as described in section 2.2, there is no reason to assume inactivation of the enzyme.

Next, we studied the effect of teasing the amoebae on squares of coverslip glass. This experiment was conducted in essentially the same way as the preceding one: Two lots of 15 amoebae were weighed and the amoebae of one lot were directly transferred into 15 microtubes with glycerol-phosphate. The amoebae of the other lot were transferred onto squares of coverslip (as described in section 2.6), teased, and then transferred to the microtubes. Incubation 2 hours at 40° and pH 7.4.

As table 2 shows, the activity per unit of r. w. was also in this experiment not more different than could be expected considering the differences between individual amoebae (compare section 2.6).

Table 2.

Effect of mechanical cytolysis on peptidase activity.

	Number of amoebae	r. w. of lot, μg	Average r. w. per amoeba, μg	Average A_{pe} per amoeba, μl HCl/hour	Average Pe μl HCl/hr. × μg
Untreated amoebae ...	15	16.43	1.10	2.77	2.52
Teased amoebae	15	14.22	0.95	2.50	2.63

It was therefore concluded that it was not necessary to tease the amoebae prior to the determination of peptidase activity.

Although the preceding experiments had demonstrated that cytolysis was not necessary to obtain satisfactory contact between the amoeba peptidase and its substrate, we were interested in connection with other studies, in the effect of the cytolyzing agent 657 L on peptidase activity (compare section 2.6). To investigate this the following experiments were run:

Effect of 657 L on amoeba homogenates: 14 amoebae were homogenized with 3 μl + 158 μl glycerol-phosphate, the homogenate was divided into equal parts, and to one part was added 5 μl of a 5 % solution of 657 L, to the other part the same amount of water. Samples of 7 μl were incubated with 7 μl substrate for 1.5 hours at 40° at pH 7.4.

Table 3.

Effect of the cytolyzing agent 657 L on peptidase activity.

	Average A_{pe} without 657 L, μl HCl/hour	Average A_{pe} with 657 L, μl HCl/hour
Homogenate I	2.69	2.90
Homogenate II	3.38	3.40

As table 3 shows for 3 experiments, there is no effect of 657 L; the difference between the two averages (4 %) is well within the limits of error.

Effect of 657 L directly on amoebae: 3 amoebae were cut into approximately equal halves by means of glass needles and then weighed. One part of each amoeba was transferred to 7 µl glycerolphosphate containing 657 L (5 % stock solution diluted 1:20) the other part to pure glycerol-phosphate. Incubation 3 hours as usual.

Table 4.

Effect of 657 L on peptidase activity in untreated amoebae.

Designation of amoebae	657 L	r. w., µg	Ape µl HCl/hour	Pe, µl HCl/hr × µg	$Q_{pe}\left(\frac{\text{blank}}{657\ \text{L}}\right)$
340 a	+	0.895	2.17	2.42	1.36
340 b	—	0.34	1.12	3.29	
342 a	+	1.43	2.38	1.67	1.32
342 b	—	0.98	2.16	2.20	
344 a	+	0.66	2.04	3.09	1.26
344 b	—	0.41	1.60	3.90	

As table 4 shows , there is in this case some inactivation of peptidase by the cytolyzing agent. This is rather surprising in view of the fact that no such effect could be observed in the case of the homogenates. Since for the purpose of the present investigation there was no need to make use of 657 L we have for the time being not followed this clue any further.

3.15. Peptidase distribution in centrifuged amoebae.

To study this, the main problem of our investigation, three different sets of experiments were run. In the first group we determined what variation in the distribution of peptidase activity was to be expected if uncentrifuged amoebae were divided in two parts. It was conceivable, though not probable, that the activity per unit r. w. would turn out to be in some way dependent on the size of the portions. In

the second group the amoebae were centrifuged before being divided, but stratification was not pushed very far, and before cutting the amoebae were allowed to re-mix partially, so that both portions obtained would contain a number of nuclei sufficient to ensure complete recovery. In the third group the amoebae were stratified as far as we dared go without causing death and cytolysis during the subsequent operations of cutting, weighing and transfer to the peptidase determination tubes.

Group 1: Uncentrifuged amoebae.

After one day's starvation the amoebae were cut with glass needles in two parts the sizes of which varied between 1:1 and 1:3. The largest part was in all cases called *a*, the other *b*. The parts were allowed to recover for about one hour, then weighed and after weighing transferred to microtubes containing the usual 7 μl of weak phosphate-glycerol and treated in the usual way.

Table 5.

Uncentrifuged amoebae, peptidase.

Designation of amoebae	1		2		3		4	
	r. w., μ g		Ape, μ l HCl / hour		Pe µl HCl / hr x µg		Qpe (a/b)	"Indirect" S.D'. of Qpe
	a	b	a	b	a	b		
154	0.73	0.65	1.65	1.55	2.26	2.38	0.95	0.046
155	0.695	0.67	1.48	1.71	2.13	2.55	0.84	0.041
168	1.29	0.52	2.00	0.71	1.55	1.37	1.13	0.079
179	0.74	0.36	2.86	1.58	3.86	4.39	0.88	0.055
180	0.51	0.22	1.90	1.01	3.72	4.6	0.81	0.086
182	0.43	0.20	1.59	0.88	3.70	4.40	0.84	0.096
183	0.39	0.24	1.92	1.13	4.92	4.70	1.05	0.084
183	0.39	0.24	1.92	1.13	4.92	4.70	1.05	0.084
184	0.49	0.455	1.98	1.88	4.05	4.13	0.98	0.055
185	0.73	0.29	2.40	1.04	3.29	3.59	0.92	0.072
186	0.98	0.425	2.86	1.26	2.92	2.96	0.99	0.054
187	0.55	0.29	1.55	0.75	2.82	2.58	1.09	0.096
188	0.53	0.27	1.63	0.80	3.08	2.96	1.04	0.089
189	0.335	0.145	1.19	0.42	3.55	2.90	1.22	0.149
196	0.335	0.315	1.08	1.12	3.22	3.56	0.91	0.082

Mean of Q_{pe}: 0.975 S. E. ($\overline{Q_{pe}}$): 0.03 S. D. (Q_{pe}): 0.12
S. D'. (Q_{pe}): 0.12 Mean of "indirect" S. D'. (Q_{pe}): 0.08.

In table 5, columns 3 a and b give the specific peptidase activities per hour and per μg of r. w. These numbers, while grouping themselves around an average

value of 3.29, show a considerable spread (1.37 to 4.92), thus expressing the variability of the species with regard to enzyme content. Column 4 shows the comparison quotients (the ratio of the specific activities of the two halves). Its average is very close to 1, which was to be expected if the distribution of peptidase throughout the amoeba was homogeneous and not dependent on the relative size of the portions. It is hardly a coincidence that the largest deviation from unity occurs in the case where the reduced weight was smallest, that is where the experimental error was probably greatest.

Group 2: Slightly centrifuged amoeba.

After one day's starvation the amoebae were centrifuged at ~1000 × g for 20–30 minutes under constant observation. After centrifugation they were removed from the cuvette and placed under a dissection microscope at room temperature. As soon as re-mixing had attained such a degree that it could be assumed that some nuclei had found their way into the presumptive light half, the amoebae were cut about midway and parallel to the planes of stratification. This usually took about 5 minutes after centrifugation had ceased. The two portions were then allowed to recover until they had completely lost their stratification and begun to form pseudopodia; this took usually about 20 minutes. During that time the animals were observed at frequent intervals to make sure that they did not cast off substantial portions of cytoplasm. If they did, the experiment was discontinued. If not, they were weighed, transferred to microtubes and the peptidase activity determined in the usual way.

In table 6 the light and heavy halves are designated “l” and “h”. In all other respects the table corresponds to table 5. The average value of the ratio l/h of the specific activities is 1.23, sufficiently different from unity to indicate that the light halves contain relatively more peptidase than the heavy ones.

Group 3: Strongly centrifuged amoebae.

The indication obtained in the experiments just reported was followed up by utilizing in this group of experiments amoebae that were more sharply stratified. The main difference consisted not in the conditions of centrifugation which were essentially the same as in the preceding group, but in the fact that no re-mixing was allowed before the cutting. For this reason the centrifuging and cutting were performed at 0° in a cold-room, but otherwise conditions were quite

Table 6.

Slightly centrifuged amoebae, peptidase.

Designation of amoebae	1 r. w., μg		2 Ape, μl HCl/hour		3 Pe μl HCl/hour × μg		4 Q_{pe} (l/h)
	h	l	h	l	h	l	
158	0.78	0.675	3.12	2.26	4.00	3.35	0.84
160	0.30	0.38	1.27	2.28	4.23	6.00	1.42
161	0.34	0.42	1.91	2.74	5.61	6.53	1.16
164	0.36	0.56	0.645	1.09	1.79	1.95	1.09
166	1.58	1.61	1.76	1.88	1.11	1.17	1.05
167	0.56	1.63	0.52	2.08	0.93	1.27	1.37
168	1.61	0.615	2.04	1.20	1.27	1.95	1.53
176	0.46	0.72	1.24	2.26	2.70	3.14	1.16
177	0.25	0.75	1.00	2.32	4.00	3.10	1.29
178	0.33	0.54	1.13	2.19	3.43	4.05	1.18
190	0.55	0.59	1.73	2.22	3.14	3.76	1.20
191	0.735	0.485	2.47	1.90	3.36	3.92	1.17
192	0.72	1.01	1.27	3.04	1.76	3.01	1.71
193	0.56	0.60	1.96	2.53	3.50	4.21	1.20
195	0.295	0.28	0.99	1.05	3.36	3.75	1.12

Mean of Q_{pe}: 1.23 S. E. (Q_{pe}): 0.05
S. D. (Q_{pe}): 0.21 S. D'. (Q_{pe}): 0.17

the same as described for the preceding group. Cutting was done immediately after removal of the amoebae from the cuvette, and was somewhat more difficult, since the freshly centrifuged amoebae were rounder and stiffer than the partially re-mixed ones. The difference in appearance of the halves was much more pronounced than in group 2, the light halves being clearer and in most cases completely free from nuclei. After cutting, both halves were allowed to recover at room temperature as previously described, then weighed and their enzyme content determined. Mortality was decidedly higher than in group 2.

As table 7 indicates the distribution of specific peptidase activity is much more unequal in this group than in group 2, the mean comparison quotient being 1.76.

3.2. Proteinase.

3.21. pH optimum.

Homogenate: 5 amoebae in 3 μl strong + 30 μl weak phosphateglycerol. 3.2 μl of homogenate + 6.5 μl substrate-buffer mixture (1:1).

Table 7.

Strongly centrifuged amoebae, peptidase.

Designation of amoebae	1 r. w., μg h	1 r. w., μg l	2 Ape. μl HCl/hour h	2 Ape. μl HCl/hour l	3 Pe, μl HCl/hour × μg h	3 Pe, μl HCl/hour × μg l	4 Q_{pe} (l/h)
286	1.54	0.49	1.59	1.47	1.03	3.00	2.91
290	1.01	0.16	2.98	0.89	2.95	5.56	1.89
293	0.915	0.26	2.92	1.41	3.20	5.43	1.70
294	1.24	0.13	2.98	0.34	2.40	2.62	1.09
295	0.71	0.17	2.88	0.875	4.06	5.15	1.27
296	1.08	0.77	2.92	4.58	2.70	5.95	2.20
297	0.81	0.36	2.96	2.21	3.66	6.15	1.68
299	1.27	0.19	4.46	1.20	3.51	6.31	1.80
300	0.81	0.10	2.97	0.42	3.67	4.20	1.15
324	0.36	0.38	1.01	1.83	2.81	4.82	1.71
350	1.15	0.36	2.61	1.57	2.27	4.36	1.92
352	1.38	0.58	2.67	2.00	1.93	3.45	1.79

Mean of Q_{pe}: 1.76 S. E. ($\overline{Q_{pe}}$): 0.14
S. D. (Q_{pe}): 0.49 S. D'. (Q_{pe}): 0.28

Every determination corresponded to 0.5 amoeba. Incubation for 6 hours at 40°.

Fig. 6 represents a condensation of three experiments of per cent of the maximum obtained. For practical reasons all subsequent experiments were performed not exactly at the pH optimum found, but at pH 3-75.

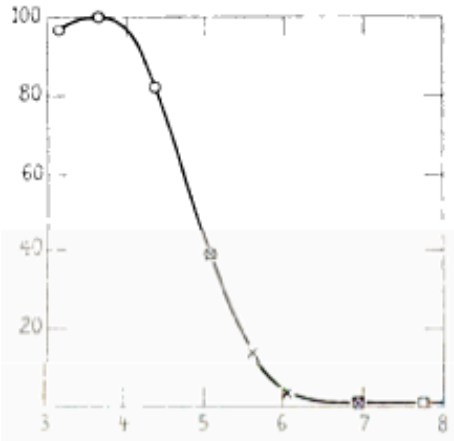

Fig. 6. p_H-optimum curve of proteinase activity. Abcissa: p_H. Ordinate: activity in per cent of maximum. ○, × and □ represent 3 different experiments. Each point is the mean of 2–3 determinations.

3.22. Relation between time and extent of cleavage.

Unlike the case of peptidase (section 3.12) the cleavage of high-molecular substrates by proteinases is usually not a linear function of cleavage time. As shown by fig. 7, the amoeba proteinase is no exception to this rule. Homogenate 10 amoebae in 3 μl

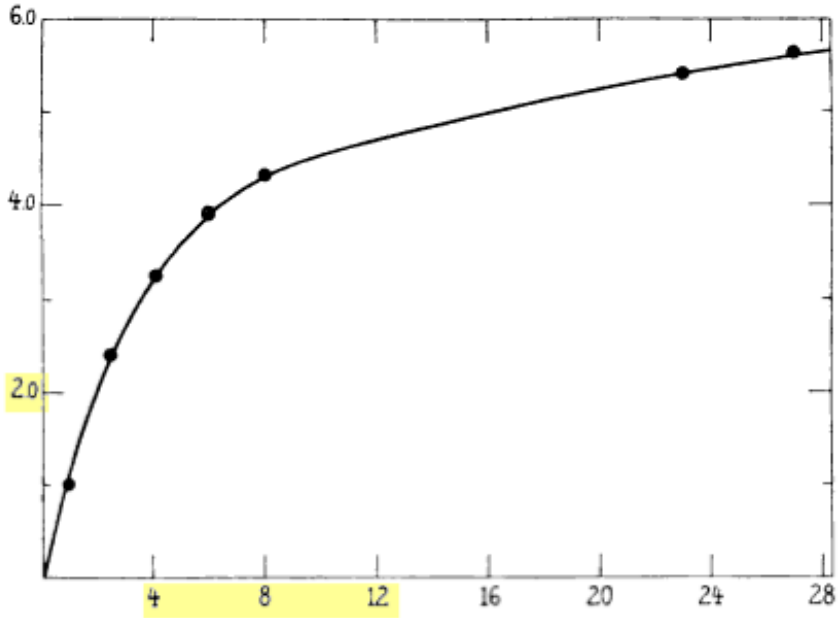

Fig. 7. Correlation between proteinase activity and time.
Abcissa: hours. Ordinate: tyrosine units.
Each point is the mean of 2 determinations.

strong + 60 µl weak phosphate-glycerol. 3.2 µl homogenate + 6.5 µl substrate-buffer (pH 3.75) per determination, corresponding to 0.5 amoeba, Incubation at 40° for the times indicated in fig. 7.

The curve shown in fig. 7 was used if different cleavages, for the sake of comparison, had to be reduced to the same cleavage time value. Small corrections of the order of a few minutes, however, as they occur in the course of routine determinations, were made assuming linear dependence for the short intervals involved.

3.23. Relation between enzyme concentration and extent of cleavage.

Homogenate: 21 amoebae in 3 µl strong + 60 µl weak phosphate-glycerol. This stock solution was diluted in suitable proportions so as to give the relative concentrations indicated on fig. 8. Concentration 1.00 corresponds to approximately 1 amoeba per sample of 3.2 µl (1 amoeba unit). Incubation 4 hours at 40°.

Fig. 8 is condensed from 4 experiments of this type. The curve thus obtained was used as a "standard curve" to convert the tyrosin equivalents obtained in the photometric measurements into "amoeba units". The latter value can be considered as a measure of the amount of proteinase present in a given sample

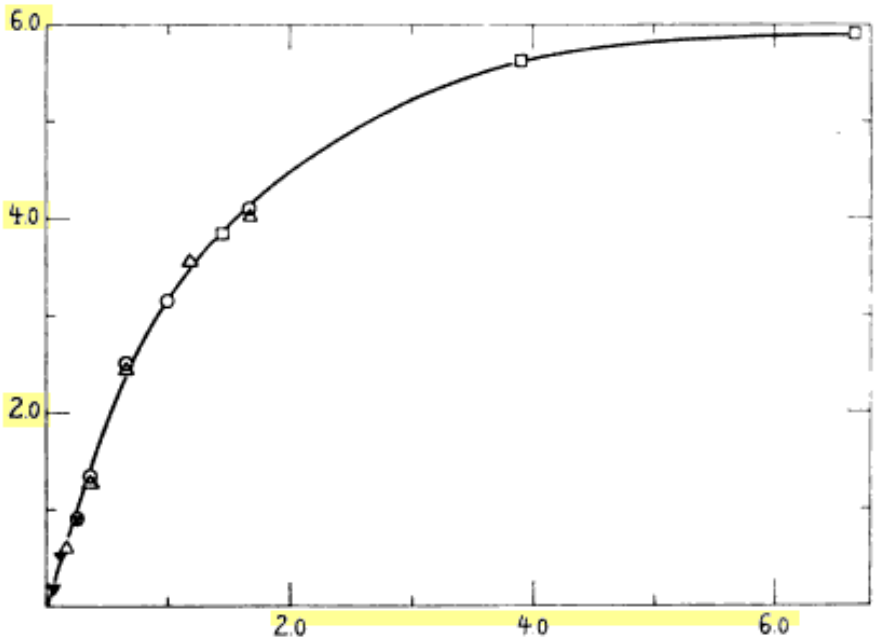

Fig. 8. Correlation between proteinase activity and enzyme concentration. Abcissa: relative enzyme concentration in amoeba units. Ordinate: tyrosine units.
▼, △, □, and ○ represent 4 different experiments.
Each point is the mean of 2–3 determinations.

3.24. Activation and inactivation.

It has been claimed (ANSON (3)) that catheptic proteinase is not activated by the usual activators if the degradation of large protein molecules is taken as a measure of activity instead of the number of peptide bonds split. Since the method here employed belongs to the former type activation was not to be expected. To make sure, however, we carried out the following experiment , designed to test the activation by cystein and inactivation by iodoacetate.

Homogenate: 18 amoebae were homogenized in 3 μl strong + 60 μl weak phosphate-glycerol. Of this stock solution samples of 17 μl were taken and to these added 3 μl 0.1 M cystein or 0.01 M iodo-acetate. 3 μl samples of these mixtures were incubated with 6.5 μl of substrate-buffer in the usual way. In the controls the addition of cystein or iodoacetate was delayed until just before the enzymatic reaction was stopped by trichloracetic acid.

Table 8 shows the result of several experiments of this kind. Every number is the average of 3 determinations. Since different homogenates were used, the absolute values cannot be compared, but only the corresponding pairs of values in the vertical columns.

Table 8.

Effect of cysteine and iodoacetate on proteinase activity.

Compound added	Average A_{pr}, without addition, amoeba units	Average A_{pr}, with addition, amoeba units
	0.72	0.76
cysteine, M: 0.49×10^{-2}	0.70	0.72
	0.73	0.82
	0.92	1.24
iodoacetate, M: 0.49×10^{-3}	1.04	1.32

It is seen that the effect of cystein undoubtedly does not exceed the errors of the method. There might be a slight activation by odoacetate, which, if real, would be rather surprising since either no effect or inactivation was to be expected. This clue was not followed as it was of no real importance for this study.

3.25. Effect of grinding and cytolysis on proteinase activity.

For the reasons given in section 2.6 we wanted to make sure that also in the case of proteinase the contact between enzyme and substrate would be sufficient under the conditions of our experiments. First we tried the effect of homogenization.

30 amoebae were weighed in lots of five. Three of the lots were used for making a homogenate in the usual manner, the rest of the amoebae were transferred singly into microtubes and the proteinase activity determined. 3 μl PRINGSHEIM solution was transferred together with each amoeba; the size of the homogenate samples was likewise 3 μl, each containing the material of approximately one amoeba.

Table 9 shows the result of the comparison. It will be seen from the last two numbers of the last column that the average specific activity of the untreated amoebae was 1.30, while the corresponding average for the homogenized amoebae was 1.95. The activity of the untreated amoebae is thus about 30 % lower. It is quite possible that this result is to be explained by the large variation of the specific proteinase activities of the individual amoebae which will be seen

Table 9.

Effect of homogenization on proteinase activity.

	Number of amoebae	Number of amoebae, per sample	r. w. of lot, µg	Average r.w. per sample, µg	Average A_{pr} per sample, amoeba units	Average Pr, amoeba am.un./µg
Untreated amoebae, lot 1	5	1	3.62	0.72	1.08	1.50
Untreated amoebae, lot 2	5	1	4.42	0.88	0.99	1.13
Untreated amoebae, lot 3	5	1	2.54	0.51	0.66	1.29
Untreated amoebae, lots 1 + 2 + 3	15	1	10.58	0.70	0.91	1.30
Homogenate	14	1.01	8.35	0.60	1.17	1.95

from table II (section 3.26). On the other hand, this result might also indicate that cytolysis plays a more important role in proteinase determinations than in the case of peptidase. In order to investigate this question further we studied the effect of the cytolyzing agent 657 L.

That the agent had no influence on the proteinase activity itself was ascertained by the following experiment:

5 amoebae were homogenized in 35 µl weak phosphate-glycerol. Two samples of 17 µl each were taken from the homogenate. To one sample was added 1 µl 657 L, to the other 1 µl of water. The proteinase activity was determined in 3 µl samples of the two mixtures. As table 10 shows, the activity was practically the same in both cases.

Table 10.

Effect of 657 L on proteinase activity.

Average A_{pr} without 657 L, amoeba units	Average A_{pr} with 657, amoeba units
0.56	0.54

Finally, the effect of cytolysis caused by 657 L was studied by means of weighed amoeba-halves, similarly to to the peptidase experiment

summarized in table 4 (section 3.14). 6 amoebae were cut in approximately equal halves and then weighed. One part of each amoeba was transferred in the usual way to proteinase substrate containing 5% 657 L, the other part to substrate containing the same amount of water. The 6 amoebae thus treated were included in table II (section 3.26). As the table shows, there was no significant difference between the halves treated with 657 L and the untreated halves. We have therefore concluded that preliminary cytolysis was unnecessary for the determination of proteinase activity.

3.26. Proteinase distribution in centrifuged amoebae.

The experiments designed to determine the distribution of proteinase activity during centrifugation were quite analogous to the peptidase experiments described in section 3.15, the only difference being that in the case of proteinase we omitted the group of slightly centrifuged animals and compared only uncentrifuged and strongly centrifuged amoebae.

Group 1: Uncentrifuged amoebae.

After one day's starvation the amoebae were cut in halves. The two parts were allowed to recover for about one hour, then weighed. The largest part was designated a, the other b. After weighing, the halves were transferred to microtubes together with 3 μl, 6.3 μl substrate were added and the samples were incubated for 2 hours at 40° and pH 3.75.

Table II shows the result, including 6 amoebae one part of which had been treated with 657 L. Since no specific effect of 657 L could be found the numbers from these amoebae were included in the calculation of those averages which concerned the whole group. The individual variation of specific proteinase activity is of the same order as in the peptidase experiments. The activity ratios of the two halves are grouped around 1, showing a somewhat higher spread than in the case of peptidase. This is probably due to the wider range of error of the proteinase determination method.

Group 2: Centrifuged amoebae.

The centrifuging and cutting was done exactly as described in section 3.15 for the strongly centrifuged amoebae. After centrifugation, cutting and recovery, the amoebae were weighed and their proteinase activity determined as described for the preceding group.

Table 11.

Uncentrifuged amoebae, proteinase.

Designation of amoebae	1		2		3		4	5	6
	r. w., μg		A_{pr}, amoeba units		P_r, am. un./μg		Q_{pr} (a/b)	Q_{pr} (657 L / blank)	"indirect" S.D′. of Q_{pr}
	a	b	a	b	a	b			
329	0.70	0.54	0.41	0.40	0.59	0.74	0.80	..	0.28
330	0.69	0.61	0.40	0.32	0.58	0.53	1.09	..	0.32
332	0.88	0.52	0.50	0.30	0.57	0.58	0.98	..	0.32
333	1.02	0.60	0.72	0.24	0.71	0.40	1.78	..	0.35
334	1.53	0.93	1.08	0.44	0.71	0.47	1.51	..	0.19
335	0.84	0.56[1])	0.40	0.46	0.48	0.82	0.59	1.69	0.26
337	1.12[1])	0.95	0.84	0.46	0.75	0.48	1.56	1.56	0.20
338	1.30	1.16[1])	1.00	1.02	0.77	0.88	0.88	1.14	0.11
361	1.36[1])	0.96	1.60	0.94	1.18	0.98	1.20	1.20	0.10
362	2.04	1.21[1])	1.96	0.64	0.96	0.53	1.81	0.55	0.14
363	1.88	1.41[1])	1.32	1.04	0.70	0.74	0.95	1.05	0.10

[1]) = 657 L added.

Mean of all Q_{pr}: 1.20. S. E. ($\overline{Q_{pr}}$): 0.12. S. D. (Q_{pr}): 0.40.
S. D′. (Q_{pr}): 0.34. Mean of "indirect" S. D′. (Q_{pr}): 0.22.
Mean of Q_{pr} (657 L/control): 1.20. Mean of Q_{pr} (a/b, no 657 L) 1.23.

Table 12.

Centrifuged amoebae, proteinase.

Designation of amoeba	1		2		3		4
	r. w., μg		A_{pr}, amoeba units		P_r, am. un./μg		Q_{pr} (l/h)
	h	l	h	l	h	l	
315	1.06	0.41	2.08	0.20	1.96	0.49	0.25
317	0.95	0.49	1.12	0.17	1.18	0.35	0.30
318	1.01	0.39	1.12	0.20	1.11	0.51	0.46
319	0.60	0.79	1.20	0.44	2.00	0.56	0.28
321	1.03	0.79	1.76	0.40	1.71	0.51	0.30

Mean of Q_{pr}: 0.32. S. E. ($\overline{Q_{pr}}$): 0.04.
S. D. (Q_{pr}): 0.09. S. D′. (Q_{pr}): 0.27.

As table 12 shows, the dislocation of proteinase brought about by centrifuging is rather great. The average ratio between the specific activities of the light and heavy halves (Q_{pr}.(l/h)) is 0.32, as compared with the value 1.20 for the corresponding ratio Q_{pr}. (a/b) in the

uncentrifuged amoebae. Therefore, even though the latter value deviates from unity by 20 %, we feel that the effect of centrifugation is sufficiently pronounced to permit the conclusion that the greater part of proteinase activity is centrifuged down into the heavy half of the amoeba.

3.3. Sources and magnitudes of error.

In experiments like ours it is rather difficult to evaluate the technical errors, as they are combined with the biological variation of the material. It is, however, possible to a certain degree to estimate and combine the errors involved in the single steps of the various operations.

3.31. Error in the determination of reduced weight.

The obvious way of determining the error of the weighing procedure is to measure its reproducibility by repeated weighings of the same object. This is not very easy in the case of amoebae. They cannot stand indefinitely the manipulations involved in weighing and there is reason to believe that invisible but measurable bits of cytoplasm are loosened in the process and are lost altogether or may stick to the balance cup, thus influencing the subsequent weighings. Also dust particles are potential sources of error. During the routine work with the diver balance we formed the impression that accidents of this kind are the main source of weighing error, much more than the actual measurement of the equilibrium pressure. The accuracy of this measurement corresponds to less than 0.005 μg r. w. with the balances used in our experiments. The actual weighing errors are greater (compare ZEUTHEN (25)).

To obtain an estimate of the overall error committed in the weighings of our amoebae we plotted the equilibrium pressures of empty balances as a function of time and determined the regression lines of the graphs so obtained by the method of the least squares. Fig. 9 shows two typical examples. The single points represent control weighings of the empty balance; in the time between these, 1-4 (generally 2) amoeba weighings had been performed.

The deviations of the single points from the regression line (especially noticeable in curve 2) permit an estimate of the overall error committed in weighing amoebae. Table 13 shows in four instances the standard deviation of the single points about the regression

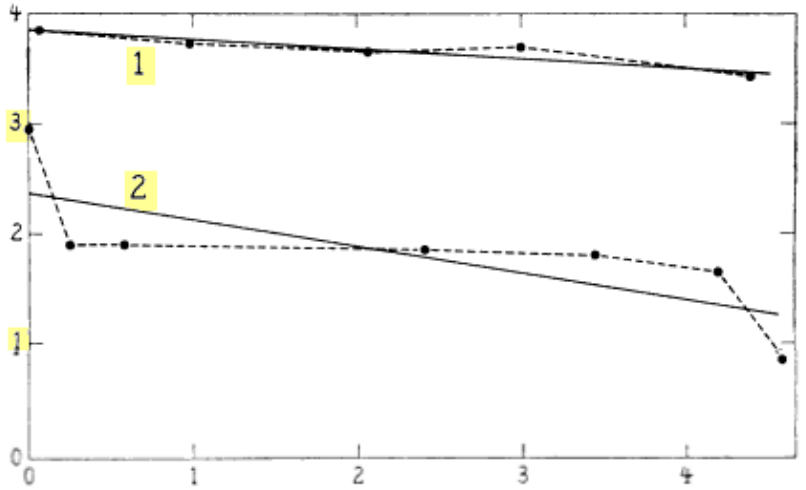

Fig. 9. Illustration of the change of equilibrium pressure of the empty diver balance.
Abcissa: hours. Ordinate: cm BRODIE's solution.
The unbroken lines represent the regression lines as calculated according to the method of the least squares. The dotted lines are the ones actually used for interpolation of the equilibrium pressure of the diver balance at the time of weighing amoebae.

line. Two of them are identical with the examples shown in fig. 9. –– The average error of 0.015 μg r. w. agrees quite well with the corresponding value (0.01) obtained by ZEUTHEN (25).

Table 13.

Standard deviation of weighings on the diver balance.

Example	1	2	3	4	Average
S. D., μg	0.015	0.006	0.002	0.035	0.015

It is probably somewhat too high, since in the actual determinations of the r. w. of amoebae the dotted lines in fig. 9 have been used for interpolation, and not the regression line. This would tend to decrease the standard deviation. Nevertheless, the value 0.015 has been used in the subsequent calculations of the compound standard deviation.

3.32. Error in the enzyme determinations.

For the reasons given before the standard deviation of the enzyme determination in a single amoeba cannot be computed directly. The best approximation is obtained

from the enzyme determinations with amoeba homogenates. We have therefore selected some of those experiments where a sufficient number (3-5) parallel determinations were run and have from these calculated an average standard deviation of the enzyme determinations. Tables 14 and 15 show the result for the peptidase and proteinase determinations.

Table 14.

Standard deviation of peptidase activity determinations (each example is calculated from 5 values).

Example	1	2	3	4	5	6	Average
S. D., μl HCl	0.10	0.15	0.16	0.10	0.11	0.11	0.12

Table 15.

Standard deviation of proteinase activity determinations (each example is calculated from 3 values).

Example	1	2	3	4	5	6	7	Average
S. D., amoeba units	0.080	0.042	0.110	0.121	0.051	0.010	0.144	0.080

The peptidase error is practically the same as found previously in applying the same method to other material. For the proteinase determination no material is available for comparison.

3.33. Standard deviation of the comparison quotients.

From the values of the comparison quotients we have calculated directly the mean, the standard deviation and the standard error of the mean (table 5, 6, 7, 11, and 12). This procedure presumes that all values of Q belong to the same statistical universe, i. e. that the variation of the deviations from the mean is merely due to chance. If we consider only uncentrifuged amoebae (table 5, and 11) we will see that the values, although being close to 1, are calculated as the ratio between two values of Pe or Pr, whose numerical values vary appreciably from one amoeba to another. One might expect that a value of Q determined as the ratio between two large numbers is more accurate than one determined from two small ones. Now, a great value for Pe or Pr implies a relatively great enzyme-activity

divided by a relatively small reduced weight; this, on the other hand, indicates a smaller error in the numerator and a greater one in the denominator, if we presume the absolute errors to be the same in all cases. The reverse is the case with small values of Pe and Pr. To get an impression of how far these trends cancel each other out, we have calculated the "relative standard deviation", S. D'. (standard deviation divided by mean), and compared these values with the "indirectly" calculated values of S. D'. obtained in the following way:

Assuming that the absolute error of the measurement was the same in all cases, and equal to the average values obtained in the two preceding sections, the standard deviations of the specific activities can be computed from the formulae:

(Ia) $$\frac{S.D.\,(Pe)}{Pe} = \sqrt{\left(\frac{S.D.\,(A)}{A}\right)^2 + \left(\frac{S.D.\,(r.w.)}{r.w.}\right)^2}$$

or

(Ib) $$S.D.'(Pe) = \sqrt{(S.D.'(A))^2 + (S.D.'(R.W.))^2}$$

where S.D'. denotes the relative standard deviation.

The relative standard deviation for the comparison quotients are obtained according to the following formula:

(2a) $$S.D.'\,(Q_{pe}) = S.D.'\left(\frac{Pe_a}{Pe_b}\right) = \sqrt{\left(S.D.'(Pe_a)\right)^2 + \left(S.D.'(Pe_b)\right)^2}$$

By inserting (Ib) in (2a) we obtain:

(2b) $$S.D.'\,(Q_{pe}) = \sqrt{\left(S.D.'(A_a)\right)^2 + \left(S.D.'(r.w._a)\right)^2 + \left(S.D.'(A_b)\right)^2 + \left(S.D.'(r.w_b)\right)^2}$$

They have been calculated for the uncentrifuged amoebae, and the values have been given in the last columns of tables 5 and 11. It will be seen that there is no correlation between the numerical size of the Pe and Pr values and the calculated relative standard deviations, a fact which justifies the method of direct calculation of S. D., as discussed in the beginning of this section. If we compare the averages of the "indirect" S. D'. values (0.077 and 0.22) with the "direct" S. D'. of the comparison quotients, namely, 0.123 and 0.314, it will be seen that these values come as close to each other as could reasonably be expected, considering the many sources of error which are not taken into account in the preceding "indirect" calculation.

In the experiments with centrifuged amoebae, only the "direct" standard deviations have been calculated (tables 6, 7, and 12). Here the centrifugation and stratification introduce a series of new factors the weight of which can scarcely be estimated by any indirect means. We shall revert to this point in the discussion. –However, it seems reasonable to assume that the total error due to technical factors must be of the same order of magnitude as in the case of uncentrifuged amoebae.

4. DISCUSSION

With regard to the distribution of peptidase activity, the present paper supports and in some respects extends, previous studies on the peptidase of *Chaos diffluens* (*Amoeba proteus*).

HOLTER and KOPAC (17) and HOLTER and DOYLE (15) had already found considerable variations of peptidase activity in single amoebae from the same culture. Since no volume or weight measurements were available, however, they could not determine whether this was only due to variations in size or also to variations in the specific peptidase activity. In the present study this question could be investigated, and it turned out (table 5) that even when the size of the amoebae was corrected for there still remained a large variation in individual peptidase activities. The same is true of proteinase (table 11), of respiratory intensity (15) and probably of many other physiological constants. The reason for this is unknown.

The data of HOLTER and KOPAC and HOLTER and DOYLE can be used for a rough comparison of peptidase activities of *Chaos diffluens* and *Chaos chaos*. The average activity of a single *Chaos diffluens* was found in the two previous papers to be about 0.08 µl 0.06 N HCl per hour. According to CHALKLEY's measurements (5) the average volume of *Chaos diffluens* in a typical culture is 1.5 X 10^{-3} µl. The peptidase activity per ul amoeba is thus 53 µl 0.06 N HCl per hour. The corresponding value for *Chaos chaos* is (table 5) 3.3 µl per µg r. w., and since, according to ZEUTHEN's measurements, 1 µg r.w. corresponds to 0.05 µl cytoplasmic volume, 1 µl *Chaos chaos* has a peptidase activity of 66 µl 0.06 N HCl per hour. The specific activities of the two species are thus certainly of the same order of magnitude. No data on *Chaos diffluens* are available for a similar comparison with regard to proteinase activity[2].

Concerning the main subject of our investigation, the question of centrifugal dislocation of enzyme activities, the results presented in tables 5-7 and 11-12 show without any doubt that peptidase and proteinase activities are located in different cytoplasmic constituents. The next question is whether we can draw any conclusions as to which cytoplasmic constituents might be involved.

As stated in the introduction, the results of earlier work on sea urchin eggs (12) and *Chaos diffluens* (17) have been taken as an indication that peptidase in these

[2] Some such data have now been procured, consult N. ANDRESEN and HOLTER: Science, in press (1949).

objects must be located in the hyaloplasm. Since then our knowledge of cytoplasmic components has been considerably widened, especially by CLAUDE's detection of the very small particles which are now most commonly designated microsomes (7, 4). The question as to whether these particles are enzyme-bearers is, as far as we can see, not yet entirely settled. With special regard to peptidase, however, attention should be drawn to the fact that BRACHET and JEENER (4) have found that at least part of the cellular peptidase in various objects (including frog's eggs) is located in microsomes. Furthermore there is no doubt that under the conditions of centrifugation employed in our experiments (both in the earlier papers quoted and in the present study) the microsomes would be found together with the hyaloplasm. In other words, in the light of subsequent developments it would be more correct to conclude from the experiments reported previously in papers (17) and (12) that peptidase may be located either in the hyaloplasm or in microsomes – or in both.

It is difficult to say whether the evidence obtained in the present study supports such an assumption. We are not able, in the case of amoebae, to measure the size of the various strata formed during centrifugation, as the stratification is not sufficiently sharp for that. Moreover, the degree of vacuolization of the cytoplasmic inclusions of the amoeba is still not very well known and therefore it is impossible to judge the degree of packing of granular material which is at least partly surrounded by vacuoles of varying size, as described by ANDRESEN (1). For these reasons we cannot, as was done in the case of the sea-urchin eggs, calculate the relative amounts of hyaloplasm and inclusion bodies present in the centrifuged portions of amoebae. Qualitatively, however, it can safely be assumed that the peptidase cannot be bound exclusively to the nuclei or to any of those granular or vacuolar inclusions that are accumulated upon centrifugation under our conditions near one of the poles of the amoeba. (For the arrangement of the various strata in the centrifuged amoeba consult fig. 2 in ANDRESEN's paper (1)). This seems to leave only the assumption that either all cytoplasmic components may carry peptidase, but in varying amounts, or that peptidase is localized in the hyaloplasm. But here we run into the difficulty that we know so little about the actual nature of the hyaloplasm. It was already found by HOLTER and DOYLE (16) that in centrifuged *Chaos diffluens* there are at least 2 zones of hyaloplasm which must have different

densities. This was confirmed for *Chaos chaos* by ANDRESEN (1) who showed that the different layers of hyaloplasm, while optically empty and undistinguishable under the microscope, reveal a different structure after fixation and staining.

As our activity values refer to units of reduced weight, it is obvious that the specific gravities of the two halves of the centrifuged amoeba will influence the numerical value of the comparison quotient. Therefore as a measure of size of centrifuged cell fragments the reduced weight cannot be regarded as ideal, as long as the specific gravities of the halves are unknown (and still more unknown are the densities of the various cytoplasmic constituents of which they are composed). In future experiments it is intended to combine the weighings with determinations of volume or specific gravity. But at present, all we can say is that the light halves must be bigger in terms of volume than indicated by their reduced weight, and that the distribution of peptidase would appear much more even, if considered in terms of volume instead of reduced weight. In this sense, therefore, there is nothing in our present experiments that would contradict the earlier studies, where the even distribution of peptidase was regarded as characteristic for this enzyme.

Even if the use of reduced weight as a quantity of reference does not enable us to draw direct conclusions as to enzyme localization, it can safely be used as a basis of comparison between different enzymes. In this respect our experiments are quite unambiguous and they show that proteinase in contradistinction to peptidase is clearly preponderant in the heavy halves. Some heavy granular materials such as the mitochondria might well be expected to behave during centrifugation in the way indicated by the distribution of proteinase activity and might therefore be suspected to act as carriers of this enzyme. In the case of liver tissue, granules of a similar type have been claimed by CLAUDE (7) to be associated with several enzymes which, however, did not include proteases. CLAUDE's claims have recently been confirmed, at least partially, by SCHNEIDER, CLAUDE and HOGEBOOM (23) and HOGEBOOM, SCHNEIDER and PALLADE (11), whereas CHANTRENNE (6) regards the question as being somewhat more complicated. Owing to the impossibility of performing exact measurements on the centrifuged amoeba we cannot, for the time being, decide with certainty whether the amoeba proteinase is associated with some particular type of granules; it might be bound to any heavy component of the hyaloplasm. All we can

say with certainty is that at least its major part must be associated with some heavy component of the amoeba cytoplasm and that this component is not the one carrying the major part of the peptidase.

From a comparison of tables 5–7 and 11–12 it follows that the relative standard deviation (S. D'.) of the comparison quotients (the ratio between the specific activities of the halves) increases upon centrifugation in the case of peptidase, but not in the case of proteinase. This is exactly what one would expect on the basis of the assumptions which we have just discussed.

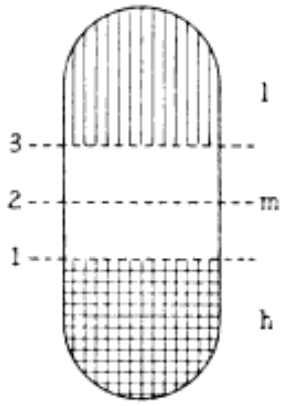

Fig. 10. Schematic drawing of a centrifuged amoeba.
h, m and l represent the 3 different zones mentioned in the text. The volumes of h, m, and l are equal. 1, 2, and 3 represent different levels of cutting.

Let us assume that the centrifuged amoeba consists of three zones of equal volumes, as shown in fig. 10. The heavy zone "h" and the light zone "l" contain most of the granular and vacuolized inclusions, while the middle zone "m" consists mainly of hyaloplasm (in the broadest sense of the term). A comparison with figs. 2 and 4–5 in ANDRESEN's paper (1) will show that this schematical picture is not too far from the actual conditions. – The average specific gravity of *Chaos* is 1.020 according to ZEUTHEN (25). Let us therefore assume that the specific gravities of the three zones are 1.010, 1.020 and 1.030 respectively, corresponding to relative reduced weights in the ratio 1:2:3.

Now let us assume that peptidase is evenly distributed throughout the non-granular and non-vacuolized material; we would then expect the middle zone to have the highest relative activity, whereas the activities of l and h would be lower, and equally so, provided their relative contents of non-active material were equal. Let us therefore assume the relative activities l:m:h to be 1:4:1. If on the basis of these assumptions we calculate the comparison quotients of the amoeba fragments obtained by cutting along the planes indicated by the dotted lines 1, 2 and 3 in fig. 10, we find the quotients l/h to be 5.0, 2.0 and 1.0. Table 16 shows a few other values of comparison quotients, corresponding to different assumptions for the relative peptidase activities.

In table 17 a few examples pertaining to proteinase have been calculated in exactly the same way, only with essentially different assumptions regarding the relative enzyme activities, taking into account the hypothesis that in this case the enzyme is bound to some cytoplasmic component which is mainly collected in the heavy third of the centrifuged amoeba.

It is thus shown by tables 16 and 17 that by making a few not unreasonable assumptions which bring the calculated values within the range of the actually observed numbers, we can account for the fact that the scattering of the comparison quotients is larger for peptidase than for the proteinase.

Table 16.

Influence of different level of cutting on Q_{pe}.

	Activity ratio l:m:h		
	1:4:1	1:3:1	1:2:1
cut 1	5.0	4.0	3.0
cut 2	2.0	2.0	2.0
cut 3	1.0	1.3	1.7

Table 17.

Influence of different level of cutting on Q_{pr}.

	Activity ratio l:m:h				
	1:4:14	1:4:16	1:4:18	1:3:16	1:5:16
cut 1	0.36	0.31	0.28	0.25	0.38
cut 2	0.37	0.33	0.30	0.29	0.38
cut 3	0.28	0.25	0.23	0.26	0.24

But we can not, for instance, decide whether in the case of peptidase the change in comparison quotients upon centrifugation is brought about exclusively by the change in specific gravity of the halves, or by a combination of this influence and an actual displacement of the enzyme[3].

With regard to the physiological functions of the two enzymes it must be concluded that they probably do not take part in the same process. In connection with their investigations on microsomes BRACHET and JEENER (4) have suggested the hypothesis that the enzymatic arsenal of these granules might be assembled as participants in the common task of protein synthesis. Any such role

[3] Note, added in proof: Some determinations of specific gravity have been carried out and have so far given average values of 1.0140 and 1.0245 for the light and heavy halves, respectively. Correcting the comparison quotients by means of these numbers, and thus referring them to units of volume instead of r.w. we find: Q_{pe} 1.76 x 0.57 = 1.0, and Q_{pr} = 0.32 x 0.57 = 0.18. This means that peptidase is evenly distributed, and proteinase still more preponderant in the heavy half, in agreement with the above considerations.

requiring association in space seems to be excluded by the results of our experiments, at least for a great part of the two enzymes concerned. From all we know one of the most characteristic properties of some peptidases is their ubiquity and it seems reasonable to assign to them a role which explains and necessitates this ubiquity, as was done by BRACHET and JEENER (4) in the hypothesis just mentioned, or by DUSPIVA (9) who makes the tentative suggestion that the function of peptidases is to break down the peptides that appear continuously during the renewal of cytoplasmic proteins. The function of the amoeba proteinase, on the other hand, must be much more specialized, but until further evidence is available we cannot say what this function might be.

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SUMMARY

By means of titrimetric and colorimetric micro-methods, the amounts of peptidase (substrate alanyl-glycine) and proteinase (substrate caseinogen) in *Chaos chaos* were determined and some properties of these enzymes investigated. It was found that cytolysis is not essential for the enzyme determination.

The enzyme contents in individual amoebae were measured and referred to units of reduced weight, as determined in ZEUTHEN'S Cartesian diver balance. It was found that the individual variations were great and of the same order of magnitude for both enzymes.

The amoebae were centrifuged in a microscope-centrifuge until the cytoplasmic components were stratified; then they were cut in two, weighed and the enzyme contents of the parts determined. It was found that peptidase remained comparatively evenly distributed during centrifugation with a tendency to be accumulated in the lighter half. Proteinase, on the other hand, was predominantly found in the heavier half, where the greater part of the granular cytoplasmic inclusions were also accumulated. From this it is concluded that peptidase and proteinase are bound, at least in part, to different cytoplasmic components.

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